PATENT CLAIMS

1. Method for identifying genes with site-specific or site preferred expression in specific target cells present in a cell environment different or not from that of their origin,
**characterized in** that the target cells initially are detected and isolated by repeated immuno-magnetic procedures in order to obtain up to 100% specific target cells before exposing the said target cells to known cell cloning procedures.

2. Method according to claim 1,
**characterized in** that the used target cells are malignant cells obtained from solid primary or recurrent tumors; and/or from metastases from such tumors to lymph nodes; and/or blood; and/or bone marrow; and/or bone tissue; and/or liver; and/or lungs; and/or central nervous system; and/or malignant pleural effusions and ascites, urine; and/or cerebral spinal fluid; and/or other organ sites.

3. Method according to claims 1-2,
**characterized in** that the malignant cells are isolated from single cell suspensions prepared from solid tumor manifestations; and/or from mononuclear cell fractions obtained from bone marrow or blood samples; and/or from cells present in other body fluids.

4. Method according to claims 1-2,
**characterized in** that the malignant cells used are *in vitro* cultivated human tumor cells; and/or human tumor cells grown in specific tissues in immunodeficient animals; and/or experimental human tumor metastases in such animals.

5. Method according to claims 1-4,
**characterized in** that RNA and/or DNA are extracted from the isolated cells.

6. Method according to claim 5,
**characterized in** that the extracted nucleic acids are used for gene cloning purposes.

7. Method according to the above claims,
**characterized in** that the said gene cloning method is the differential display or the subtractive hybridization approaches, or any other procedure that can be used to identify genes with differential expression.

8. Method according to claim 7,
**characterized in** that amplified cDNAs obtained from malignant cells selected from different sites are studied and compared on sequencing gels, and where those with interesting site-specific or site-preferenced patterns are sequenced and identified.

9. Method according to claim 8,
**characterized** in that the expression patterns of identified gene sequences are studied on material obtained from all relevant tumor cell sites described in claims 1-4.

10. Method according to the preceding claims,
**characterized in** that previously unknown genes identified in preceding claims are used for gene therapy purposes, and/or as targets for procedures aimed at altering or inactivating the genes or their products.

11. Use of the method according to claim 1, to obtain specific gene sequences and their expression products in target cells present in cell environments different or not from their origin.

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference | FOR FURTHER ACTION | See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416) |
|---|---|---|
| Fod4 im | | |
| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
| PCT/NO97/00083 | 25/03/1997 | 26/03/1996 |
| International Patent Classification (IPC) or national classification and IPC | | |
| C12Q1/68 | | |
| Applicant | | |
| FODSTAD, Oystein et al | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 16/10/1997 | 29.04.98 |
| Name and mailing address of the IPEA/ | Authorized officer |
| European Patent Office<br>D-80298 Munich<br>Tel. (+49-89) 2399-0, Tx: 523656 epmu d<br>Fax: (+49-89) 2399-4465 | Goetz, M<br>Telephone No. (+49-89) 2399-8697 |

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NO97/00083

## I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

| | |
|---|---|
| 1-7 | as originally filed |

**Claims, No.:**

| | | | | |
|---|---|---|---|---|
| 1-11 | as received on | 09/04/1998 | with letter of | 07/04/1998 |

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 1-11 |
| | No: | Claims | |
| Inventive step (IS) | Yes: | Claims | 1-11 |
| | No: | Claims | |
| Industrial applicability (IA) | Yes: | Claims | 1-11 |
| | No: | Claims | |

2. Citations and explanations

**see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**see separate sheet**

## REASONED STATEMENT

1. None of the documents cited in the IPER relates to the use of the combined techniques

   a) immuno-magnetic techniques for the isolation of specific target cells,
   b) cell cloning procedures,

   for identifying unknown genes with site-specific or site-preferred expression at the mRNA levels.

   Hence, claims 1 - 11 meet the novelty requirements pursuant to Art. 33(2) PCT.

2. Although the manipulative technique listed under a) and b) above are known from the prior art as represented by

   **D1 = WO95/24648**
   **D2 = WO94/07139**

   (see **D1**, page 5/lines 14 - 16, page 15/lines 20-26, claims 1 - 13 and 16; see **D2**, page 3/last paragraph, claims 1 - 14), and e.g. **Science 257, 1992, p. 967 - 971**, (cited in the application), the present inventors apparently were the first to establish that the preferential appearance of metastatic tumor cells in certain tissues is due to an up- and down-regulation mechanism of specific genes in the tumor cells, reflected by differential mRNA expression.

   Hence, the use of known techniques a) and b) for identifying unknown genes participating in such mechanisms involves an inventive step.

   Claims 1 - 11 therefore meet the requirements pursuant to Art. 33(3) PCT.

→

## CERTAIN DOCUMENTS

As the present application is fully entitled to the claimed priority date of 26/03/96, the interfering documents, i.e.

**Proc.Annu.Meet.Am.Assoc.Cancer Res., 37:A607, 1996**
and **Proc.Annu.Meet.Am.Assoc.Cancer Res., 37:A555, 1996**

are not considered to represent state of the art relevant to presently claimed subject-matter.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/68, C12N 5/00, G01N 33/543
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CANCERLIT, EMBASE, WPI

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9524648 A1 (FODSTAD), 14 Sept 1995 (14.09.95), page 4, line 2 - page 8, line 9 -- | 1-9 |
| X | WO 9407139 A1 (FODSTAD), 31 March 1994 (31.03.94), See pages 4-8 and 16 and claim 14 -- | 1-9 |
| X | National Library of Medicine, file Medline, Medline accession no. 95268055, Van Epps DE et al: "Harvesting, characterization, and culture of CD34+ cells from human bone marrow, peripheral blood, and cord blood", Blood Cells 1994;20(2-3):411-23 -- | 1-6 |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 July 1997 | 08 -07- 1997 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Carl-Olof Gustafsson<br>Telephone No. +46 8 782 25 00 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Dialog Information Service, file 154, Medline, Dialog accession no. 07911215, Medline accession no. 94231000, Wixler V. et al: "Isolation and quantification of class 1 MHC gene mutants in mouse T cells by immunoselection with a magnetic cell sorter (MACS)",J Immunol Methods May 2 1994, 171 (1) p121-30 | 1,5,6 |
| X | Dialog Information Service, file 154, Medline, Dialog accession no. 06887713, Medline accession no. 92084142, Kappes DJ et al: "A novel method for generating stabel high-level transfectants involving direct immunomagnetic selection for cell-surface epitopes: exprression of HLA class-II genes in HeLa cells", Gene Dec 15 1991, 108 (2) 245-52 | 1,4-6 |
| X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01195522, Cancerlit accession no. 96133084, Yaremko ML et al: "Immunomagnetic separation can enrich fixed solid tumors for epithelial cells", Am J Pathol; 148(1):95-104 1996 | 1-3,5,7 |
| X | National Library of Medicine, file Medline, Medline accession no. 95325659, Griwatz C et al: "An immunological enrichment method for epithelial cells from peripheral blood", J Immunol Methods 1995 Jun 28;183(2):251-65 | 1-9 |
| P,X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01193425, Cancerlit accession no. 96709393, Ree AH et al: "Identification of site specific gene expression in metastatic breast cancer cells (Meeting abstract)", Proc Annu Meet Am Assoc Cancer Res; 37:A607 1996 | 1-9 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01192976, Cancerlit accession no. 96653514, Brandt B. et al: "Isolation of single cancer cells and coherent cell sheets from peripheral blood of cancer patients with reference to shed cytokeratin 8/18 and erbB-2 oncoprotein fragments (Meeting abstract)", Proc Annu Meet Am Assoc Cancer Res; 37:A555 1996 | 1-9 |
| A | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 00864158, Cancerlit accession no. 92257480, Gazitt Y et al: "Expression of N-Myc, and MDR-1 Proteins in Newly Established Neuroblastoma Cell Lines: A Study by Immunofluorescence Staining and Flow Cytometry", Cancer Res; 52(10):2957-65 1992 | 1-9 |

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 5 : | A1 | (11) International Publication Number: WO 94/07139 |
|---|---|---|
| G01N 33/53, C12Q 1/00<br>C12N 5/00 | | (43) International Publication Date: 31 March 1994 (31.03.94) |

(21) International Application Number: PCT/NO93/00136

(22) International Filing Date: 10 September 1993 (10.09.93)

(30) Priority data:
PCT/NO92/00151 14 September 1992 (14.09.92) WO
*(34) Countries for which the regional or international application was filed:* NO et al.

(71)(72) Applicants and Inventors: FODSTAD, Øystein [NO/NO]; Frits Kiers v. 28, N-0383 Oslo (NO). KVALHEIM, Gunnar [NO/NO]; Åsstubben 13, N-0381 Oslo (NO).

(74) Agent: ONSAGERS PATENTKONTOR AS; P.O. Box 265 Sentrum, N-0103 Oslo (NO).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: IMPROVED METHOD FOR DETECTION OF SPECIFIC TARGET CELLS IN SPECIALIZED OR MIXED CELL POPULATION AND SOLUTIONS CONTAINING MIXED CELL POPULATIONS

(57) Abstract

The invention relates to a method for detecting specific target-cells in a simple and time saving way, using paramagnetic particles, antibodies recognizing the Fc portions of target-cell associating antibodies and target-cell associating antibodies directed to specific antigen determinants in the target-cell membranes. Incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method. The method can further be used for isolation of the target-cells by magnetic field application and a kit for performing the method according to the invention is described.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | |
|---|---|
| AT | Austria |
| AU | Australia |
| BB | Barbados |
| BE | Belgium |
| BF | Burkina Faso |
| BG | Bulgaria |
| BJ | Benin |
| BR | Brazil |
| BY | Belarus |
| CA | Canada |
| CF | Central African Republic |
| CG | Congo |
| CH | Switzerland |
| CI | Côte d'Ivoire |
| CM | Cameroon |
| CN | China |
| CS | Czechoslovakia |
| CZ | Czech Republic |
| DE | Germany |
| DK | Denmark |
| ES | Spain |
| FI | Finland |
| FR | France |
| GA | Gabon |
| GB | United Kingdom |
| GN | Guinea |
| GR | Greece |
| HU | Hungary |
| IE | Ireland |
| IT | Italy |
| JP | Japan |
| KP | Democratic People's Republic of Korea |
| KR | Republic of Korea |
| KZ | Kazakhstan |
| LI | Liechtenstein |
| LK | Sri Lanka |
| LU | Luxembourg |
| LV | Latvia |
| MC | Monaco |
| MG | Madagascar |
| ML | Mali |
| MN | Mongolia |
| MR | Mauritania |
| MW | Malawi |
| NE | Niger |
| NL | Netherlands |
| NO | Norway |
| NZ | New Zealand |
| PL | Poland |
| PT | Portugal |
| RO | Romania |
| RU | Russian Federation |
| SD | Sudan |
| SE | Sweden |
| SI | Slovenia |
| SK | Slovak Republic |
| SN | Senegal |
| TD | Chad |
| TG | Togo |
| UA | Ukraine |
| US | United States of America |
| UZ | Uzbekistan |
| VN | Viet Nam |

Improved method for detection of specific target cells in specialized or mixed cell population and solutions containing mixed cell populations

The present invention relates to an immunomagnetic method for detection of specific target cells in cell populations and solutions of cell populations. The invention also relates to a kit for performing the method in different cell populations.

In biology, biochemistry and adjacent fields it is considerable need for methods in which chemical entities are linked together. Such methods have an increasing importance in research regarding both normal and abnormal cell populations. Especially when solid supports are used cells can be immobilized, detected and isolated and purified. Furthermore, the cell content may be examined using a range of sofisticated methods. It is also of importance to be able to isolate the cells in viable forms.

Affinity binding is a sofisticated way of linking chemical/biochemical entities together. In such a method a pair of binding partners, which for example are attached to the substances to be linked, bind to each other when brought in contact. One of the binding partners in such a linkage may be represented by a molecule on the cell surface. Several such binding partner systems are known, such as antigen- antibody, enzyme- receptor, ligand- receptor interactions on cells and biotin- avidin binding, of which antigen-antibody binding is most frequently used. A hapten/anti-hapten binding pair method has also recently been suggested (PCT/EP90/01171).

When such methods are used for isolation of specific cells, which are the subject for further various examinations, it is necessary to reverse the linkage without producing destructive effects on the binding partners, which ideally should recover their function upon returning to the original conditions. This is not always the case, although it is proposed a method for

adequately cleaving antigen/anti-antigen and hapten/anti-hapten linkages (PCT/EP91/00671, PCT/EP90/01171).

Methods are known in which one of the binding partners is attached to an insoluble support, such as paramagnetic particles, and by which isolation of target cells in a mixed cell population is performed as negative isolation or positive isolation. In a negative isolation procedure the unwanted cells can be removed from the cell preparation by incubating the cells with antibody-coated particles, specific for the unwanted cells. Following the incubation the cell/antibody/particle-complex can be removed using the particles, leaving the wanted target cells behind. This result is often not satisfactory, since the wanted cells are left in the cell population, more or less purified, and also since the intention of the isolation procedure is to examine and/or perform further studies on the specific target cells. Attempts have been made to use particles for positive isolation, in which the wanted target cells are removed from the mixed cell population. These procedures have, however, been directed to certain target cells and are not suited for all target cell systems. A positive isolation procedure involving the hapten/anti-hapten linkage system has recently been proposed (PCT/EP90/01171) and also a method for isolating haemopoietic progenitor cells from bone marrow (PCT/EP90/02327). The latter is directed to use a combination of positive and negative selection for the purpose of isolating and possibly growing specific cells, i.e. haematopoietic progenitor cells, in the bone marrow, and is dependent upon removal of the particles.

PCT/EP90/01171 relates to a method of connecting target cells to an insoluble support by using the abilities of hapten, anti-hapten antibodies and anti-cell antibodies to bind to each other, thus constructing a linkage between the insoluble support, i.e. particle, and the target cell, consisting at least of hapten and anti-hapten antibody or combinations of hapten and anti-hapten antibodies and anti-anti-hapten antibodies or secondary anti-cell antibodies. The later

cleavage of the complex is performed by again exposing it to hapten or hapten analogue. Thus the constructed link always consists of hapten in addition to 1 or more elements. The method is directed to unspecified target cells and is directed to isolation of target cells and release of the insoluble support.

There is a need for a simple linkage to connect a target cell to an insoluble support, which do not involve compounds of a rather unspecified haptene-group, and which is directed to detection of specific target cells, with a minimum of unspecific cell association and which render unnecessary a later cleavage between the insoluble support and the specific target cell.

Thus the object of the present invention is to detect for diagnostic purposes specific target cells when used in a blood and bone marrow, without the problem with unspecific binding to normal cells. It represents a sensitive detection method for a variety of cell types, such that a high number of cells can be readily screened in the microscope and the procedure is rapid and simple. Furthermore, the present method can be used for isolation of cells for biochemical, biological and immunological examination, and for studying of specific genes at the nucleotide or protein level, in addition to culturing the cells, without the need for cleaving the cell-particles complex. A further object of the invention is to provide a kit for performing the method as characterized in the claims.

The intensions of the inventions are obtained by the method and kit characterized in the enclosed claims.

The method for immunomagnetic detection of target cells in a mixed cell population and physiological solutions containing cell populations is suitable for detection, but may also be used in positive isolation of specific types of both normal cells and patogenic cells. The method creates a linkage between a specific target cell and an insoluble support, such as

paramagnetic particles , which consists of one or two elements. The particle is either coated with an anti-cell antibody of murine or human origin, directed to the specific antigen determinants in the membranes of the wanted target-cells, or the particles are coated with a polyclonal anti-mouse or anti-human antibody capable of binding to the Fc-portions of the specific anti-cell antibody directed to the antigen determinants in the target-cell membranes. Instead of using the polyclonal anti-mouse/anti-human antibody for coating the particles, a monoclonal rat anti-mouse/anti-human antibody may be used. This last antibody, due partly to its monoclonal origin, may provide a more specific binding to the anti-cell antibody and reduce the risk for possible cross-reactions with other cells in solutions, such as blood. Furthermore, incubation of the cell suspension with a mild detergent and/or second set of antibodies or antibody fragments, prelabeled or not with fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or certain enzymes allowing visualization, will dramatically increase the specificity of the method.

In the following a more detailed disclosure of the method is presented, using cancer cells as the target-cells for detection and possible isolation. The method is, however, not limited to cancer cells and the disclosure shall not limit the method to this particular field of use, since the method is suitable within a range of cytological research areas.

In the management of cancer patients, the staging of the disease with regards to whether it is localized or if metastatic spread has occurred to other tissues, is of utmost importance for the choice of therapeutic alternative for the individual patient. Malignant cells spread by direct invasion into the surrounding tissue, through the lymphatics or by the distribution of tumor cells in the blood to distant organs, including the bone marrow and the central nervous system and the cerebrospinal fluid.

Detection of metastatic tumor cells has, until recently, relied on morphological methods using light and electron microscopy on biopsied tumor specimens, on smears of bone marrow and peripheral blood, and on slides prepared after cytosentrifugation of various body fluids. Since the advent of monoclonal antibodies recognising antigens predominantly expressed on the surface of different types of malignant cells, the identification of metastatic cells has, to an increasing extent, also involved immunocytochemistry and immunofluorescence. Thus, slides prepared from biopsied tumors or cytosentrifugates have been treated with monoclonal antibodies, and the binding of these to the tumor cells is visualized colorimetrically or by fluorescence. The latter method requires the use of a fluorescence microscope, alternatively preparing a cellsuspension an use a flow cytometer.

The previous methods suffer from limited sensitivity and/or specificity, and is usually laborious and time consuming, also requiring a high degree of expertise. Flowcytometric examiniations also involve expensive equipment.

The morphological methods for the detection of tumor cells in blood and bone marrow are much less sensitive than methods involving immunocytochemistry and immunofluorescence (Beiske et al., Am. J. Pathology 141 (3), September 1992). Also the latter methods are, however, inadequate in cases where the tumor cells represent less than 1 % of the total number of nucleated cells. Flow cytometry may provide better sensitivity than the methods involving the use of a microscope, but requires the availability of a high number of cells, and also involves several technical difficulties. Thus, aggregation of cells may cause problems, and the method does not provide possibilities to distinguish between labeled tumor cells and unspecifically fluorescing normal cells.

The invention allows for a very sensitive detection of, for example, metastatic tumor cells, since a high number of cells

can readily be screened in the microscope and the attached magnetic beads are easily recognisable. The monoclonal antibodies used bind with sufficient specificity to, for example, tumor cells and not to other cells than the target-cells present in mixed cell suspensions, like blood, bone marrow, and in other tumor manifestations, such that all cells with attached beads represent the target-cells. In addition, the procedure is rapid and simple, and can be performed by any investigator with access to a conventional microscope.

The novel method involves the binding of monoclonal antibodies, e.g. of murine or human origin, that specifically recognize antigens present on tumor cells, and not on the normal cells in question, or for other purposes to specified subpopulations of normal cells, to paramagnetic particles, either directly or to beads first covered with antibodies specifically recognizing the respective antibodies, or the Fc-portion of IgG antibodies, that bind to the tumor cells. The cell binding antibodies may be of the IgG or IgM type or being a fragment of ab IgG or IgM. Examples of used anti-target-cell antibodies may be those directed against groups of antigen determinants, for example CD56/NCAM antigen (MOC-1), Cluster 2 epithelial antigen (MOC-31), Cluster 2 (MW~40kD) antigen (NrLu10) (Myklebust et al. Br. J. Cancer Suppl. 63, 49-53, 1991), HMW-melanoma-associated antigen (9.2, 27) (Morgan et al., Hybridoma, 1, 27-36, 1981), 80kD, Sarcoma-associated antigen (TP1 & TP3) (Cancer Res. 48, 5302-5309, 1988), mucin antigens (Diel et al., Breast Cancer Res. Treatm., 1991), or EGF-receptor antigen (425.3) (Merck), in addition to the anti-pan-human antibody (Bruland et al., unpublished), which is suitable for detecting human cells among animal cells. The 425.3 antibody is directed towards antigens in both normal and malignant cells. Antibodies can furthermore be directed against growth factor receptors, for example EGF-receptor, PDGF (A and B) receptor, insuline receptor, insuline-like receptor, transferrin receptor, NGF and FGF receptors, group of integrins, other adhesion membrane molecules and MDR proteins in both normal cells and abnormal cells, and antigens present on subpopulations of normal cells,

in addition to oncogenic products, expressed on the membranes of normal and malignant cells and on malignant cells alone, for example Neu/erb B2/HER2. As for the malignant cells, these may be breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma, cancer cells of the gastrointestinal tract and the reticuloendothelial system, or the target-cells may be associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune, gastrointestial, genitourinary, reticuloendothelial and other disorders. Furthermore, the malignant cell population may be located in bone marrow, peripheral blood, come from pleural and peritoneal effusions and other body fluid compartments, such as urine, cerebrospinal fluid, semen, lymph or from solid tumors in normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissue, the central nervous system, prostatic gland, skin and mucous membranes. A more complete list of the antigen determinants and the corresponding antibodies or antibody fragments used in the present improved method is presented in Table 1.

The method comprises attachment of the antibodies directly to the paramagnetic particles, or the attachment can take place by attachment to surface-bound antibodies, such as polyclonal anti-mouse antibodies, monoclonal rat anti-mouse antibodies or monoclonal anti-human antibodies, specifically recognizing the Fc-portion of the said individual antibodies. The antibody-coated paramagnetic beads are then mixed with the suspension of cells to be examined and incubated for 5-10 min to 2 h, preferably for 30 min at 0-25°C, preferably at 4°C, under gentle rotation. The present method may also be performed in a changed order of steps, in that the free target-cell antibodies are added to the cell suspension, incubated for 5-10 min to 2h, preferably 30 min, at 0-20°C, preferably 4°C, under gentle rotation. The paramagnetic particles, precoated with anti-mouse or anti-human antibodies are then added to the incubated cell suspension, as described above, and the resulting suspension subjected to a further incubation of 5-10 min to 2h, preferably 30 min, at 0-25°C, preferably 4°C under gentle agitation.

Samples of the cell suspension are then transferred to a cell counting device, and the fraction of cells with attached beads relative to the total number of cells is determined under light microscopy. The number of antibody-coated beads added to the cell suspension should be between 0.5-10 times the number of target cells. When this number is unknown, the amount of coated beads added should be 1-10 % of the total number of cells.

For specific purposes, and in the cases where the density of the target-cells is low, for example malignant cells, or the target-cells represent a very low fraction of the total number of cells (≤ 1%), the target cells can be positively separated from non-target cells in a magnetic field. The isolated target cells, can then be enumerated microscopically and the fraction of target cells relative to the total number of cells in the initial cell suspension can be calculated. Moreover, the target-cells may be characterized for the presence of specific biochemical and biological features. Of particular importance will be the use of such cells for studies in molecular biology. In contrast to the above cited methods of the prior art, the present method allows studies and growth of the target-cells without performing a cleavage of the paramagnetic particle-target cell linkage. For several purposes it is of interest to examine specific genes in a pure population of target cells at the DNA, mRNA and protein level, both in tumor biopsies as well as in tumor cells present in blood, bone marrow and other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from otherwise normal tissues and organs, for example liver, lymph nodes, spleen, lung, pancreas, bone tissues, central nervous system, prostatic gland, skin and mucous membranes, and in other areas of cytological research activity.

With the methods of prior art, signals obtained on Southern, Northern and Western blots represent the normal cells as well as the tumor cells in the biopsy. If a single cell suspension is first prepared from the tumor material, and the tumor cells are then positively immunomagnetically detected and separated, any gene studies performed on this material would represent the

target-cells only. This also relates to for example malignant cells present in mammalian tissues, for example in bone marrow, peripheral blood, pleural and peritoneal effusions, and other body fluids, for example urine, cerebrospinal fluid, semen and lymph. Studies involving polymerase chain reaction (PCR) methodology will also gain in specificity and reliability when performed on pure tumor cell populations obtained by the new method.

The application of the new method steps may differ depending on type of tissues to be examined.

a) Tissue from solid or needle tumor biopsies is prepared mechanically or with mild enzymatic treatment into a single cell suspension, to which the primary, specific antibodies or antibody fragments are added directly or after washing the cell suspension with phosphate buffered saline or culture medium with or without serum, such as fetal calf serum, bovine, horse, pig, goat or human serum.

b) If the material is a sample of pleural or ascitic effusion, cerebrospinal fluid, urine, lymph or body fluids such as effusions in the joints of patients with various forms of arthritis, the specific antibodies or antibody fragments are either added to the samples directly, or after centrifugation with or without washings before or after the cells in the samples are spun down and brought back into suspension.

c) If the material consists of blood or bone marrow aspirate, the mononuclear cell fraction is isolated by gradient sentrifugation on e.g. Lymphoprep before washing, resuspension, and addition of the appropriate antibodies or antibody fragments.

The procedure conditions for a) and b) are established, as exemplified by results obtained in successful experiments as those described below.

For c) the results have been found to be influenced by a high number of factors which have been examined in detail. Among these are antibody concentration, the ratio of the number of paramagnetic particles versus number of cells, incubation times and volumes, type of incubation medium, and the pH level. The particle to mononuclear cell ratio in all experiments should be in the range of 0.5/1 - 2/1, depending on the binding affinity of the primary specific antibodies or fragments.

A major problem has been unspecific attachment to normal blood or bone marrow cells of particles coated with either sheep or rat anti-mouse antibodies alone, or in addition with the specific antibodies. Experiments have shown that the unspecific binding is equally high without the presence of the specific antibodies, indicating that the problem is not caused by cross-reactivity of the targeting antibodies to normal cells. The possibility that the less than optimal specificity could be caused by ionic binding has been ruled out. Another possibility was that subpopulations of normal cells of the B-lineage might adhere to the particle-antibody complexes. However, immunomagnetic removal of B-cells from the cell suspension before adding the specific antibodies/antibody-particle complexes did not improve the specificity of the latter.

The problem with the procedure used on isolated mononuclear fractions of bone marrow and peripheral bllod, that some non-target cells might also bind paramagnetic particles, has been circumvented or overcome. Thus with sheep-anti-mouse antibody coated particles alone or with specific antibodies the number of particles unspecifically attached to a low fraction mononuclear blood or bone marrow cells was reduced from an average of 10 to about 1 and in paralell the fraction of normal cells with particles decreased from 1-2% to 0.5-1% or less.

Evidence has been obtained that the problem may be caused by hydrophobic forces associated with the antibodies bound to the paramagnetic particles. Methods for reducing this hydrophobicity is thus claimed. One such method is

preincubation of the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, for example Tween 20 in concentrations of less than 0.1% for 30 minutes at 4°C. When possible selection of the target cells is warrented, the cell suspension should contain a low concentration of the detergent, e.g. 0.01% of Tween 20. In several experiments this procedure has almost eliminated or dramatically reduced the problem of unspecific binding seen with the mononuclear cell fractions from blood or bone marrow.

The other improvement which, if found warrantied, may be used together with the detergent step as follows:

After incubation of the cell suspension with the primary antibodies or antibody fragments and the antibody-coated paramagnetic particles as described in previously, the cell suspension is incubated with a second set of antibodies or antibody fragments directed against other extracellular or against intracellular determinants of the target cells, with our without pretreatment with cell fixatives such as formaldehyde or alcohols. These antibodies or their fragments should have been prelabeled by fluorescent agents, metallocolloids, radioisotopes, biotin-complexes or enzymes like peroxidase and alkaline phosphatase, allowing visualization by per se known methods in the microscope and/or a suitable counting device.

The target cells will both be visualized with the latter method and have bound particles to their surface, and can thus be enumerated.

To simplify the distinction between non-target and target cells, the cell suspension can before the second visualization step either be subjected to cytospin centrifugation or portions of the suspension are attached to caoted glass slides on which the particle-bound cells will be spread out in a thin layer, facilitating the recognition of the double-"stained" cells.

For use in the new procedure, kits will contain for example precoated paramagnetic particles prepared for each monoclonal antibody. In another embodiment the kits contain paramagnetic particles pre-coated with IgG isotype specific anti-mouse or anti-human antibody as one part of it, and different target cell-associated, for example tumor cell, antibodies as another part. In a third embodiment the kit contains paramagnetic particles precoated with specific anti-Fc antibodies, such as polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-mouse, or anti-human antibodies, capable of binding to the Fc-portion the target-cell associating antibodies, bound to specific anti-target-cell antibodies. In a further embodiment the kit contains other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target-cells, where said antibodies or antibody fragments are conjugated to peroxidase, alkaline phosphatase, or other enzymes, together with relevant substrates to such enzymes, or where said antibody or antibody fragment is bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

The present method will in the following be illustrated by model experiments, examples of the usefulness of the new method and examples of practical applications. These examples shall not be regarded as in any way limiting the invention.

Model experiments:

1. Binding of antibody-bead complexes to tumor cell lines with the new procedure:

To determine antibody concentrations and optimal conditions for the binding of antibody-paramagnetic particle complexes to tumor cells, a large panel of cancer cell lines was used. The paramagnetic beads were bound to the cells, either by coating the specific antibodies to sheep-anti-mouse antibody (SAM)-coated paramagnetic particles, or by first incubating the cells with the specific antibodies, washing, followed by a second

incubation with SAM-coated particles. The results of these experiments are given in Tables 2a and 2b, in which + indicates binding of several beads to all cells, (+) indicates either a lower number of beads bound to each cell, or that not all the tumor cells had beads attached to their surface, whereas - reflects no binding, and (-) indicates very weak binding.

2. For detection of tumor cells in the mononuclear fraction of bone marrow or peripheral blood, model experiments were performed where specific antibodies and SAM-coated paramagnetic particles were added either to such mononuclear cells or to a cell suspension where a different number of cancer cells from in vitro cultivated cell lines were added to said mononuclear cells. In some experiments, either the mononuclear cells, or the malignant cells were prestained with a fluorescent dye, to be able to distinguish beteween the two types of cells. In all experiments, non-binding primary antibodies, and/or sheep-anti-mouse antibody-coated beads were used separately as controls.

Table 2a

| Antibodies | | Cell lines | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MCF-7 | SKBR3 | T47D | MDA231 | MDA435 | DU145 | FMEX-1 | LOX |
| | | | | | | | | | |
| NrLu10 | IgG2b | | + | + | (+) | (+) | + | | |
| Moc31 | IgG1 | + | + | + | (+) | (+) | + | | |
| Moc1 | IgG1 | | | (+) | (+) | + | | | |
| 12H12 | IgG1 | | + | + | | + | + | | |
| 2E11 | IgG3 | + | + | + | | + | + | | |
| 5A6 | IgG1 | | (+) | + | | | | | |
| 5F2 | IgM | | | (+) | | | | - | |
| CC3 | IgG2a | - | - | - | | | | (+) | |
| CC1 | IgM | | | - | | | | | |
| CU18 | IgG1 | - | - | - | | | | | |
| CU46 | IgG1 | (+) | - | - | | | - | - | - |
| 7F11 | IgG1 | - | - | + | | | | | |
| D7 | IgG3 | | | (+) | | | (-) | - | 50%+ |
| E4SF | IgG1? | | + | + | | | | - | + |
| 425-3 | | | | + | | | | + | + |
| 9.2.27 | | | | | | | - | - | - |
| MUC18 | | | - | | | | | + | |
| 2g12 | IgG1 | | | | | | | + | |
| 4b7 | IgG1 | | | | | | | | |
| BM2 (=2F11) | | | | | | | | | |
| BM7 (=7F11) | | | | | | | | | |
| TP-3 | | | | | | | | | |
| TP-1 | | | | | | | | | |
| CEA | | | | | | | | | |
| GINTES | IgG | | | | | | | | |
| 3C9 | IgM | | | | | | | | |
| HH8 | IgM | | | | | | | | |
| 5F4 | IgM | | | | | | | | |
| 3F1 | IgG1 | | | | | | | | |

Table 2b

| Antibodies | | Cell lines | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PM1 | MA-11 | CRL1435 | CRL1740 | H-146 | Colo205 | 786-0 | WIDR |
| NrLu10 | IgG2b | + | + | + | + | + | + | - | |
| Moc31 | IgG1 | + | + | + | + | + | + | + | + |
| Moc1 | IgG1 | | | | | + | - | | |
| 12H12 | IgG1 | + | + | (+) | | - | - | - | |
| 2E11 | IgG3 | (+) | + | - | + | - | - | - | |
| 5A6 | IgG1 | + | + | | | | | | |
| 5F2 | IgM | | | | | | | | |
| CC3 | IgG2a | | | | | - | | - | |
| CC1 | IgM | | | | | (+) | | - | |
| CU18 | IgG1 | | | | | - | | - | |
| CU46 | IgG1 | | | | | - | | - | |
| 7F11 | IgG1 | (+) | + | - | | - | - | | - |
| ID7 | IgG3 | | | | | - | | | - |
| E4SF | IgG1? | + | + | + | + | - | - | | - |
| 425-3 | | | | | | | | | |
| 9.2.27 | | | | | | | | | |
| MUC18 | | - | | | | | | | |
| 2g12 | IgG1 | | | | | - | | - | |
| 4b7 | IgG1 | | | | | - | | - | |
| BM2 (=2F11) | | + | + | | | | | | |
| BM7 (=7F11) | | + | | | | | | | |
| TP-3 | | | | | | | | | |
| TP-1 | | | | | | | | | |
| CEA | | | | | | | | | |
| GINTES | IgG | | | | | + | | | - |
| 3C9 | IgM | | | | | - | | | - |
| HH8 | IgM | | | | | - | | | - |
| 5F4 | IgM | | | | | - | | | - |
| 3F1 | IgG1 | | | | | - | | | - |

In several experiments some unspecific binding to the mononuclear cells was observed, which was found to be unrelated to the nature of the specific antibody, and which was equally pronounced with SAM-coated particles alone. The magnitude of this unspecific binding varied from almost 0 to a level between 0.5-2%. This unspecific binding was almost eliminated by mild treatment with detergent, (Tween 20) performed to reduce the problem of hydrophobic cell interactions.

EXAMPLES OF THE USEFULNESS OF THE NEW PROCEDURE

1. Detection of micrometastic neoplastic disease in blood and marrow

Early and reliable diagnosis of spread of cancer cells to blood and/or bone marrow has become increasingly important for the choice of optimal therapy, possibly curative in many types of cancer, including carcinomas, as described in application Example 1. Similar procedures for malignant melanoma, sarcoma, neuroblastoma and several other cancers have been established or are under development.

2. Detection of malignant cells in pleural or ascitic effusions, and in urine

The nature of such effusions may represent an important diagnostic problem, particularly when a low number of cancer cells are present together with normal reactive or epithelial cells. In several cases a definite diagnosis has been rapidly made with the new method, in cases where conventional cytological examination has been negative or inconclusive. A similar advantage can be found in cases of cancer in the kidneys or in the urinary tract and bladder.

3. Detection of neoplastic cells in the cerebrospinal fluid

As the systemic treatment of many cancer types have improved, the frequency of cases with symptom-giving brain metastases have significantly increased, and in parallell with this, the necessity for early detection of such spread. With the use of the new procedure even a low number of malignant cells can easily be identified, permitting intervention with therapeutic alternatives at an early stage of intracranial tumor manifestations.

4. Diagnosis of cancer in biopsied tissue

When cancer is suspected, and tissue biopsies are obtained by surgical procedures or by e.g. needle biopsies, a much more simple and rapid diagnosis can be made with the new method, used on prepared cell suspensions, compared to conventional morphological or immunohisto- or cytochemical procedures.

Distinction between several alternative cancers can be made by the use of the appropriate antibodies.

5. Identification of prognostic indicators

Since the expression of several membrane molecules have been shown to correlate with progression of the malignant disease in several cancers, the present method can be used to identify prognostic indicators, for example as described in application Example 2.

6. Identification of cells indicative of specific diseases or of disease progression or state

In various types of rheumatoid diseases (such as rheumatoid arthritis), as well as in allergic, autoimmune, and cardiovascular diseases, identification of the systemic or local presence of specific subpopulations of cells is important

for diagnosis and for determining the stage of the disease. Rapid detection of such cell populations with the new method is therefore of considerable diagnostic and therapeutic importance.

7. Detection of subpopulations of normal cells

For several purposes, it will be important to detect the fraction of a particular subpopulation of normal cells in a population. This applies e.g. to liver biopsies where the identification of cells expressing the biliar epithelial antigen, may be of importance. Similarly, the identification, and possible isolation of specific endothelial cells from a cell suspension prepared from various normal tissues may be warranted.

Several of the cell membrane molecules mentioned in sections 1-6 may also be used as targets for immunotherapy with several types of activiated killer cells or e.g. with immunotoxins. The identification with the new method of expression of such molecules is, therefore, also of value for determining in which cases such types of therapy should be used.

Examples of a practical application of the method:

Example 1

To diagnose spread of cancer cells in blood and/or bone marrow at an early stage, we have used in the new procedure the MOC-31, NrLu10, BM2, BM7, 12H12, and MLuC1 anti-carcinoma antibodies to determine whether or not micrometastatic disease from breast, lung, colorectal, and prostate cancer might be sensitively identified in such body fluids. The successful results with these antibodies have significant clinical implications.

Example 2

The expression of serveral cell membrane molecules have been shown to correlate with progression of the malignant disease in

several types of cancer. The detection of binding of such antibodies to respective antibodies can therefore be used to obtain information of high prognostic value. Among such antigens are a high number of adhesion molecules, carbohydrate antigens, glycolipids, growth factor receptors and carcinoma markers listed below. We have, with the new procedure identified the binding of particle-antibody complexes to CD44-variants, E-cadherin, $Le^y$, CEA, EGF-r, transferrin receptor, MUC-1 epitope, LUBCRU-G7 epitope, prostate cancer antigen, UJ13A epitope, $ß_2$-microglobulin, HLA-antigens, and apoptosis receptor.

Example 3

Two litres of pleural diffusion from a patient supposed to suffer from malignant melanoma was obtained. After centrifugation, the cells were suspended in a volume of 2 ml of RPMI with a 10% fetal calf serum, incubated with 9.2.27 anti-melanoma antibody (10 µg/ml) at 4°C for 30 min, washed and again incubated with Dynabeads SAM M450/IgG2A at 4°C for 30 min. The cell suspension was then examined under a microscope for determining the fraction of cells with paramagnetic cells attached to their surface. The diagnosis of malignant melanoma was confirmed, as about 10% of the cells had a significant number of particles rosettes.

Example 4

Biopsied tissue was obtained from a subcutaneous tumor in a case with clinical indications of either small cell lung cancer or a malignant melanoma. A single cell suspension was prepared from the biopsy, divided in 2 fractions, one incubated with the 9.2.27 anti-melanoma antibody, and the other with MOC-31 anti-carcinoma antibody (both at 10 µg/ml). The incubation was similar to that used in the example above. None of the cells incubated with the melanoma antibody bound any beads, whereas all tumor cells incubated with MOC-31 were positive.

Example 5

Biopsied tissue from a patient suspected to have malignant melanoma was examined by preparing single cell suspension, incubating with 9.2.27 anti-melanoma antibody, and then following the procedure as above. Most of the cells were positive with a high number of particle-rosettes attached to their membranes.

Example 6

A pleural effusion from a breast cancer patient was studied to examine whether tumor cells could be detected in the fluid. One litre of the fluid was centrifuged, the cells resuspended, and in separate vials incubate with each of 3 different anti-carcinoma antibodies (MOC-31, 2E11, 12H12). After completing the procedure as in the previous example, it was found that most of the cells bound to antibody-coated particles in all 3 cases.

Example 7

A bone marrow suspension obtained from a breast cancer patient was studied to examine whether micrometastic tumor cells could be present. After the preparation of mononuclear cells, these were incubated with the same 3 anti-carcinoma antibodies used in the example above, but in this case the antibodies were first attached to Dynabeads SAM IgG paramagneteic particles. After 1 incubation with these directly coated particles, the cell suspension was examined in the microscope, and a high number of cells were found positive with a number of particle-rosettes attached to their membrane.

Similar experiments have been performed in a number of pleural or ascitic effusion and bone marrow from patients with breast cancer.

Example 8

T47D human breast carcinoma cells were incubated for varying lenghts of time with Hoechst fluoresence dye, and the viability of the labeled cells was checked. Varying numbers of labeled breast carcinoma cells were then added to $1 \times 10^6$ bone marrow

cells obtained from healthy volunteers. In different experiments, different concentrations of paramagnetic, monodisperse particles (Dynabeads P450) coated with individual anticarcinoma antibodies (NrLu10, MOC31, or 12H12) were added. After incubation for 30 min on ice, samples of the different test tubes were examined in a counting chamber under light and fluorescence microscopy. When the ratio of tumor cells/total nucleated cells was low, the cell suspension was subjected to a magnetic field and the cells with particles attached were isolated before examined in the microscope. It was found that at an optimal ratio of 1-10 paramagnetic beads per tumor cell in the cell mixture, all the tumor cells had from 2-15 beads attached to their surface. The sensitivity of the detection method was close to one target-cell per $10^4$ nucleated cells. In control experiments with labeled tumor cells using antibodies known to have some cross-reativity to normal cells, this cross-reactivity was confirmed with the antibody-coated paramagnetic particles. In experiments with beads without tumor-associated antibody coating, none of the target cells bound any beads.

Similar experiments have been performed both with other breast cancer lines and a small cell lung cancer cell line. Similar sensitivity and specificity were obtained in these experiments.

Example 9

Pleural and ascites fluid from patients with breast cancer and ovarian carcinoma were sentrifuged, the same coated paramagnetic particles used in Example 1 were added, incubated and concentrated in a magnetic field before the suspension was examined under light microscopy. Typically, cells that had the clear morphological features of tumor cells had beads attached, whereas none of the few normal cells bound the antibody-coated beads. In two cases with pleural effusion, an independent morphological examination did not reveal the presence of any tumor cells, whereas a significant number malignant cells were detected by the use of anibody-coated beads. In some cases, tumor cells were separated in a magnetic field and transferred

to tissue culture flasks containing growth medium specially prepared for growing breast cancer cells, in attempts to establish permanent cell lines from these cultures. In parallel, cells from the malignant effusions were cultivated directly without positive selection with magnetic beads. In the latter cases, no cell line could be established, whereas in more than 50 % of the cases where positively selected tumor cells had been used, cell lines were successfully established.

Example 10

In some cases, bone marrow and peripheral blood obtained from patients with breast cancer were examined with the present procedure by adding antibody-coated paramagnetic beads, incubating for 30 min at 4°C and concentrating in a magnetic field and by examining the suspension under light microscopy. In both cases binding of the paramagnetic beads to tumor cells, representing 0,1-1 % of the nucleated cells in the bone marrow and blood was detected, cells that could not be identified by any other method.

Example 11

Antibodies against certain growth factor receptors or other gene products expressed on the surface of specific cell populations may be used to identify and positively select these cells. Beads coated with anti-transferrin receptor antibodies, used in the novel method according to the present invention were shown to represent a rapid, simple and sensitive method for identification of cells expressing the transferrin-receptor.

Example 12

For various purposes isolation of specific populations of normal cells is warranted. Endothelial cells lining the capillary or small vessels in normal or tumorous tissue could be positively selected from cell suspensions prepared from the relevant tissues. The procedure involved the use of beads coated with antibody directed against structures expressed on

the endothelial cells, but not on the other normal cells in the cell mixture.

Example 13

Human cells injected into immunodeficient rodents was shown to be present in cell suspensions prepared from tumor xenografts and from various host organs/tissues by employing magnetic particles coated with an anti-pan human antibody.

Table 1

LIST OF RELEVANT ANTIGENS AND EXAMPLES OF ASSOCIATED ANTIGEN-BINDING ANTIBODIES

| ANTIGENS | MONOCLONAL ANTIBODIES |
|---|---|
| **Adhesion molecules** | |
| Fibronectin receptor ($\alpha5\beta1$ integrin) | Pierce 36114, BTC 21/22<br>Calbiochem 341649 |
| Integrin $\alpha3\beta1$ | M-Kiol 2 |
| Vitronectin receptor ($\alpha v\beta3$ integrin) | TP36.1, BTC 41/42 |
| Integrin $\alpha2$ | Calbiochem 407277 |
| Integrin $\alpha3$ | Calbiochem 407278 |
| Integrin $\alpha4$ | Calbiochem 407279 |
| Integrin $\alpha5$ | Calbiochem 407280 |
| Integrin $\alpha V$ | Calbiochem 407281 |
| Integrin $\beta2$ | Calbiochem 407283 |
| Integrin $\beta4$ | Calbiochem 407284 |
| GpIIbIIIa | 8221 |
| ICAM-I (CD54) | C57-60, CL203.4, RR 1/1[1] |
| VCAM-1 | Genzyme 2137-01 |
| ELAM-1 | Genzyme 2138-01 |
| E-selectin | BBA 8 |
| P-selectin/GMP-140 | BTC 71/72 |
| LFA-3 (CD58) | TS 2/9 |
| CD44 | BM 1441 272, 25.32 |
| CD44-variants | 11.24, 11.31, 11.10 |
| N-CAM(CD56) | MOC-1 |
| H-CAM | BCA9 |
| L-CAM | BM 1441 892 |
| N-CAM | TURÁ-27 |
| MACAM-1 | NKI-M9 |
| E-cadherin | BTC 111, HECD-1, 6F9 |
| P-cadherin | NCC-CAD-299 |
| Tenascin | BM 1452 193,<br>Calbiochem 580664 |
| Thrombospondin receptor (CD36) | BM 1441 264 |
| VLA-2 | A1.43 |
| Laminin receptor | |
| HNK-1 epitope | HNK-1 |
| **Carbohydrate antigens** | |
| T-antigen | HH8, HT-8 |
| Tn-antigen | TKH6, BaGs2 |
| Sialyl Tn | TKH-2 |

Table 1 (cont.)

| | |
|---|---|
| Gastrointestinal cancer associated antigen (M_200kD) | CA 19-9 |
| Carcinoma associated antigen | C-50 |
| $Le^a$ | MLuC1, BR96, BR64 |
| di-$Le^a$, tri-$Le^a$ | B3 |
| Dimeric $Le^a$ epitope | NCC-ST-421 |
| H-type 2 | B1 |
| CA15-3 epitope | CA15-3 |
| CEA | I-9, I-14, I-27, II-10, I-46, |
| | Calbiochem 250729 |
| Galb1-4GlcNac (nL4,6,8) | 1B2 |
| H-II | BE2 |
| A type 3 | HH8 |
| Lacto-N-fucopentanose III (CD15) | PM-81 |
| **Glycolipids** | ME 36.1, R24 |
| $GD_3$ | ME36.1, 3F8, 14.18 |
| $GD_2$ | 38-13 |
| $Gb_3$ | M2590 |
| $GM_1$ | MKI-8, MKI-16, |
| $GM_2$ | 1D7, F12 |
| $FucGM_1$ | |
| **Growth factor receptors** | 425.3, 2.E9, 225 |
| EGF receptor | BM 1378 988, 800 E6 |
| c-erbB-2 (HER2) | Genzyme 1264-00 |
| PDGFα receptor | Sigma P 7679 |
| PDGFβ receptor | OKT 9, D65.30 |
| Transferrin receptor | BM 1198 637 |
| NGF receptor | BM 1295 802, BM 1361 937 |
| IL-2 receptor (CD25) | BM 428 616, 14 A3, ID9.3D6 |
| c-kit | GEnzyme 1995-01, PAL-M1 |
| TNF-receptor | |
| NGF receptor | |
| **Melanoma antigens** | 9.2.27, NrML5, 225.28, |
| High molecular weight antigen (HMW 250.000) | 763.74, TP41.2, IND1 |
| Mw105 melanoma-associated glycoprotein | ME20 |
| 100 kDa antigen (melanoma/carcinoma) | 376.96 |
| gp 113 | MUC 18 |
| p95-100 | PAL-M2 |
| Sp75 | 15.75 |
| gr 100-107 | NKI-bereb |
| MAA | K9.2 |
| M_125kD (gp125) | Mab 436 |
| **Sarcoma antigens** | TP-1, TP-3 |
| TP-1 and TP-3 epitope | |

Table 1 (cont.)

| | |
|---|---|
| $M_r$200kD | 29-13, 29.2 |
| $M_r$160kD | 35-16, 30-40 |
| Carcinoma markers | |
| MOC-31 epitope (cluster 2 epithelial antigen) | MOC-31, NrLu10 |
| MUC-1 antigens (such as DF3-epitope (gp290kD)) | MUC-1, DF3, BCP-7 to -10 |
| MUC-2 and MUC-3 | PMH1 |
| LUBCRU-G7 epitope (gp 230kD) | LUBCRU-G7 |
| Prostate specific antigen | BM 1276 972 |
| Prostate cancer antigen | E4-SF |
| Prostate high molecular antigen $M_r > 400$kD | PD41 |
| Polymorphic epithelial mucins | BM-2, BM-7, 12-H-12 |
| Prostate specific membran antigen (Cyt-356) | 7E11-C5 |
| Human milk fat globulin | Immunotech HMFG-1, 27.1 |
| 42kD breast carcinoma epitope | B/9189 |
| $M_r > 10^6$ mucin | TAG-72, CC-49, CC-83 |
| Ovarian carcinoma OC125 epitope ($m_r$ 750 kD) | OC125 |
| Pancreatic HMW glycoprotein | DU-PAN-2 |
| Colon antigen Co17-1A ($M_r$37000) | 17-1A |
| G9-epitope (colon carcinoma) | G9 |
| Human colonic sulfomucin | 91.9H |
| $M_r$300kD pancreas antigen | MUSE11 |
| GA 733.2 | GA733, KS1.4 |
| TAG 72 | B72.3, CC49, CC83 |
| Undefined | Oat1, SM1 |
| Pancreatic cancer-associated | MUSE 11 |
| Pancarcinoma | CC49 |
| Prostate adenocarcinoma-antigen | PD 41 |
| $M_r$150-130kD adenocarcinoma of the lung | AF-10 |
| gp160 lung cancer antigen (Cancer Res. *48*, 2768, 1988) | anti gp160 |
| $M_r$92kD bladder carcinoma antigen | 3G2-C6 |
| $M_r$600kD bladder carcinoma antigen | C3 |
| Bladder carcinoma antigen (Cancer Res. *49*, 6720, 1989) | AN43, BB369 |
| CAR-3 epitop $M_r > 400$kD | AR-3 |
| MAM-6 epitope (C15.3) | 115D8 |
| High molecular ovarian cancer antigen | OVX1, OVX2 |
| Mucin epitope Ia3 | Ia3 |
| Hepatocellular carcinoma antigen $M_r$900kD | KM-2 |
| Hepernal epitope (gp43) Hepatocellular carc. ag | Hepema-1 |
| O-linked mucin containing N-glycolylneuraminic acid | 3E1.2 |
| $M_r$48kD colorectal carcinoma antigen | D612 |
| $M_r$71kD breast carcinoma antigen | BCA 227 |
| 16.88 epitope (colorectal carcinoma antigen | 16.88 |
| CAK1 (ovarian cancers) | K1 |
| Colon specific antigen p | Mu-1, Mu-2 |
| Lung carcinoma antigen $M_r$350-420kD | DF-L1, DF-L2 |

Table 1 (cont.)

| | |
|---|---|
| gp54 bladder carcinoma antigen | T16 |
| gp85 bladder carcinoma antigen | T43 |
| gp25 bladder carcinoma antigen | T138 |
| Neuroblastoma antigens | |
| Neuroblastoma-associated, such as UJ13A epitope | UJ13A |
| Glioma antigens | |
| Mel-14 epitope | Mel-14 |
| Head and neck cancer antigens | |
| M_18-22kD antigen | E48 |
| HLA-antigens | |
| HLA Class 1 | TP25.99 |
| HLA-A | VF19LL67 |
| HLA-B | H2-149.1 |
| HLA-A2 | KS1 |
| HLA-ABC | W6.32 |
| HLA-DR, DQ, DP | Q 5/13, B 8.11.2 |
| $\beta_2$-microglobulin | NAMB-1 |
| Apoptosis receptor | |
| Apo-1 epitope | Apo 1 |
| Various | |
| Plasminogen activator antigens & receptors | Rabbit polyclonal |
| p-glycoprotein | C219, MRK16, JSB-1, 265/F4 |
| cathepsin D | CIS-Diagnostici, Italy |
| biliary epithelial antigen | HEA 125 |
| neuroglandular antigen (CD63) | ME491, NKI-C3, LS62 |
| CD9 | TAPA-1, R2, SM23 |
| pan-human cell antigen | pan-H |

CLAIMS

1. Improved method for detecting specific target cells in cell suspensions of mixed cell populations and in fluid systems containing mixed cell populations, and in single cell suspensions prepared from solid tissues, characterized by comprising the following steps:

1.1. coating, by a per ce known procedure, paramagnetic particles or beads with either, a) antibodies, or antibody fragments directed against membrane structures specifically expressed on target-cells and not on non-target-cells in the cell mixture or;
b) antibodies, preferably polyclonal anti-mouse or monoclonal rat anti-mouse antibodies or anti-human antibodies, capable of binding to the Fc-portions of the said antibodies, directed against the membrane structures; and

1.2.1. mixing the target-cell-associating antibodies (murine or human) which is attached to the said particles or beads, or attached to the beads pre-coated with anti-mouse or antihuman antibodies recognizing the Fc-portions of the target-associating antibodies, with the cell suspension containing the target-cells, or,

1.2.2. mixing free target-cell-associating antibodies with the cell suspension containing the target cells and incubate this mixture for 5-10 min to 2 h, preferably 30 min, at a temperature between 0°C and 20°C, preferably 4°C under gentle rotation, and;

1.3. incubating the mixture of the cell suspension and target-associating antibodies attached to paramagnetic particles or beads (1.2.1), or paramagnetic particles or beads, precoated with anti-mouse or anti-human antibodies recognizing the Fc-portion of the target-associating antibodies, to the mixture of incubated free target associating antibody and cell suspension (1.2.2.), and incubating, for 5-10 min to 2 h,

preferably 30 min, at a temperature between 0°C and 25°C, preferably 4°C, under gentle rotation, and;

1.4.1. if the target cell population is contained in blood or bone marrow aspirates the hydrophobic forces associated with antibody-coated particles are reduced by pre-incubating the antibody-coated particles and the cell suspension with mild detergents in suitable concentrations, e.g. Tween 20 in concentrations less than 0.1% for 30 min at 4°C, and/or

1.4.2. by incubating the cell suspensions, untreated or pretreated with formalin, alcohol or other fixatives, with other antibodies or antibody fragments binding to extracellular or intracellular molecules present in the target cells and the antibodies used are labeled in advance by peroxidase, alkaline phosphatase, or other enzymes permitting visualization of the binding by addition and incubation with relevant substrates, or

1.4.3. the antibody fragments are biotinylated and the binding visualized when adding the incubating with avidin complexed to peroxidase, alkaline phosphatase, or other enzymes, with addition and incubation with relevant substrates, or

1.5.1. subjecting the incubated paramagnetic particle-antibodies-cell mixture (1.3) to a magnetic field if the density of target-cells is low, or if the ratio of target cell/total cells in the cell mixture is low (≤ 1 %) and then examining and counting the stained or unstained particle-target-cell complexes in the cell suspension, using a micropscope and/or a suitable cell/particle counting device, or,

1.5.2. examining and counting the target-cells in the incubated mixture of paramagnetic particles, antibodies and cell mixture (1.3), or in the case when the antibodies or antibody fragments are conjugated to non-paramagnetic particles that can be visualized directly because of colour or through enzymatic activation, using a microscope and/or a suitable cell/particle

counting device if the ratio of target-cells/total cells in the cell suspension is adequate (> 1 %).

2. Method according to claim 1, characterized by directing the antibody or fragments thereof against the antigens in normal, living cells, such as liver hepatocytes, Kupffer cells and endothelial cells type 1 and 2 and Clara cells of the lung, endothelial cells of specific organs, pancreatic exocrine and endocrine cells, kidney tubule cells, bladder epithelial cells, brain glial and ependymal cells, bladder and prostate epithelial cells, ciliated cells of airways, different subpopulations of mucosal cells in the gastrointestinal tract, pituitary cells, and other endocrine cells in various hormone-producing organs.

3. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody which is reactive with antigens present on subpopulations of normal cells and oncogenic products expressed on the membrane of normal tissue cells.

4. Method according to one of the preceding claims, characterized by using as the said positive selecting antibody, an antibody which is directed against growth factor receptors on the membrane of normal cells, for example the EGF-receptor, PDGF (A and B) receptor, insuline receptors, insuline-like receptors transferrin receptor, NGF and FGF receptors.

5. Method according to one of the preceding claims, characterized by using an antibody directed against the group of integrins and other adhesion membrane molecules, and MDR proteins in normal cells.

6. Method according to one of the preceding claims, characterized by directing the antibody or fragments thereof against antigen or receptors in cells with abnormal developmental patterns, preferably such as primary and metastatic cancer cells.

7. Method according to one of the preceding claims, characterized by using as the said target-cell associating antibodies, antibodies of the IgG isotype, or $F(ab')_2$ or F(ab) fragments, or IgM, or fragments of IgM.

8. Method according to one of the preceding claims, characterized by preparing the mentioned cell suspension from mixed cell populations comprising mammalian tissues, for examples human bone marrow and peripheral blood, from pleural and peritoneal effusions, other body fluids, for example urine, cerebrospinal fluid, semen, lymph, or from solid tumors in normal tissues and organs, for example liver, lymphatic nodes, spleen, lung, pancreas, bone tissue, central nervous system, prostatic gland, skin and mucous membranes.

9. Method according to one of the preceding claims, characterized by that the antibody or antibody fragments is directed against groups of antigen determinants, such as those listed in the Table 1 of the specification.

10. Method according to one of the preceding claims, characterized by using as the said target-cell antibody an antibody or antibody fragment which is directed against growth factor receptors and oncogene products expressed on the membrane of malignant cells, for example insuline receptors, insuline-like receptors and FGF receptors in addition to those listed in Table 1 of the specification.

11. Method according to one of the preceding claims, characterized by using an antibody or antibody fragment directed against the group of integrins, other adhesion membrane molecules and MDR proteins in abnormal cells as listed in Table 1.

12. Method according to one of the preceding claims, characterized in that the used antibodies, antibody fragments

or combinations of these are directed to the antigen determinants as listed in Table 1 of the specification.

13. Method according to one of the preceding claims, characterized by using as the said antibody an antibody which is reactive with antigens present on abnormal cells, for example breast, ovarian and lung carcinoma cells, melanoma, sarcoma, glioblastoma and cancer cells of the gastrointestinal and genitourinary tract, and of the reticuloendothelial system, and/or target-cells associated with non-neoplastic diseases, such as cardiovascular, neurological, pulmonary, autoimmune gastrointestinal, genitourinary, reticuloendothelial and other disorders.

14. Use of the detection method according to one of the preceding claims, for isolation of target-cells, whereby the complex of cells and the paramagnetic particles are exposed to a magnetic field and the resulting magnetically aggregated cells are further subjected to biological, biochemical and immunological examinations, including also characterisation of specific genes at the DNA, mRNA and protein level, including polymerase chain reaction (PCR) and reverse transcriptase PCR.

15. Use of the method for detection of specific target-cells according to one of the preceding claims, whereby it is established in vitro cell cultures from the separated paramagnetic particle-target-cell-complexes, and/or for inoculation into immunodeficient animals, preferably to establish human tumor xenografts in the said animals.

16. Kit for performing the method according to one of the preceding claims, characterized by that it comprises;
1, specific antibodies or antibody fragments directed to the antigen receptors on the wanted target-cells, where said antibody or antibody fragment is bound or can be bound to included paramagnetic particles, without removing their antigen-binding ability, and/or

2, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc portions of the target-cell associating antibodies, and specific free target-cell antibodies, and/or

3, paramagnetic particles precoated with specific anti-Fc antibodies, preferably polyclonal anti-mouse, or monoclonal rat anti-mouse, or anti-human antibodies, capable of binding to the Fc-portions of the target-cell associating antibodies, bound to specific anti-target-cell antibodies, and/or

4, other specific antibodies or antibody fragments directed against antigens/receptors within or on the wanted target cells, where said antibodies or antibody fragments are conjugated to biotin, peroxidase, alkaline phosphatase, or other enzymes, or where said antibodies or antibody fragments are bound to non-paramagnetic particles with specific colours or with bound enzymes such as peroxidase and alkaline phosphatase.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 93/00136

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: G01N 33/53, C12Q 1/00, C12N 5/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOTECHNOLOGY

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO, A1, 9204961 (IMMUNICON CORPORATION), 2 April 1992 (02.04.92), see examples 2-4 and in particular col. 14, lines 62-68 | 16 |
| Y | -- | 1-16 |
| Y | WO, A1, 9109938 (HOLMES MICHAEL JOHNET AL), 11 July 1991 (11.07.91), see the examples | 1-16 |
| X | -- | 16 |
| X | WO, A1, 9101368 (HOLMES MICHAEL JOHN ET AL), 7 February 1991 (07.02.91) | 16 |
| | -- | |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 December 1993 | 29 -12- 1993 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Carl-Olof Gustafsson<br>Telephone No. +46 8 782 25 00 |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 93/00136

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | EP, A2, 0403960 (BEHRINGWERKE AKTIENGESELLSCHAFT), 27 December 1990 (27.12.90) -- -------- | 1-16 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

27/11/93

International application No.
PCT/NO 93/00136

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A1- 9204961 | 02/04/92 | AU-A- 8548591 | 15/04/92 |
| | | US-A- 5200084 | 06/04/93 |
| WO-A1- 9109938 | 11/07/91 | AU-A- 7069791 | 24/07/91 |
| | | EP-A- 0507839 | 14/10/92 |
| WO-A1- 9101368 | 07/02/91 | AU-A- 6035090 | 22/02/91 |
| EP-A2- 0403960 | 27/12/90 | AU-A- 5717990 | 20/12/90 |
| | | CA-A- 2019217 | 19/12/90 |
| | | DE-A- 3919923 | 20/12/90 |
| | | JP-A- 3041098 | 21/02/91 |

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C12N 5/00, G01N 33/543 — A1

(11) International Publication Number: **WO 97/36004**

(43) International Publication Date: 2 October 1997 (02.10.97)

(21) International Application Number: PCT/NO97/00083

(22) International Filing Date: 25 March 1997 (25.03.97)

(30) Priority Data:
961221 26 March 1996 (26.03.96) NO

(71)(72) Applicant and Inventor: FODSTAD, Øystein [NO/NO]; Frits Kiærs vei 28, N-0383 Oslo (NO).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ENGEBRÅTEN, Olav [NO/NO]; Haneborgsveien 43A, N-1470 Lørenskog (NO). REE, Anne, Hansen [NO/NO]; Kirkeveien 87A, N-1344 Haslum (NO). HOVIG, Johannes, Eivind [NO/NO]; Sognsveien 31, N-0851 Oslo (NO).

(74) Agent: ONSAGERS PATENTKONTOR A/S; P.O. Box 265 Sentrum, N-0103 Oslo (NO).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: IMMUNO-MAGNETIC CELL SEPARATION USED IN IDENTIFICATION OF GENES ASSOCIATED WITH SITE-PREFERENCED CANCER METHASTASIS FORMATION

(57) Abstract

Method for identifying genes with site specific or site preferred expression in target cells that are initially detected and isolated by repeated immuno-magnetic procedures. The purified target cells are then exposed to known cloning procedures. Preferred target cells are malignant cells, e.g. metastatic cells. Gene cloning methods may include the differential display or substractive hybridization approaches.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | |
|---|---|
| AL | Albania |
| AM | Armenia |
| AT | Austria |
| AU | Australia |
| AZ | Azerbaijan |
| BA | Bosnia and Herzegovina |
| BB | Barbados |
| BE | Belgium |
| BF | Burkina Faso |
| BG | Bulgaria |
| BJ | Benin |
| BR | Brazil |
| BY | Belarus |
| CA | Canada |
| CF | Central African Republic |
| CG | Congo |
| CH | Switzerland |
| CI | Côte d'Ivoire |
| CM | Cameroon |
| CN | China |
| CU | Cuba |
| CZ | Czech Republic |
| DE | Germany |
| DK | Denmark |
| EE | Estonia |
| ES | Spain |
| FI | Finland |
| FR | France |
| GA | Gabon |
| GB | United Kingdom |
| GE | Georgia |
| GH | Ghana |
| GN | Guinea |
| GR | Greece |
| HU | Hungary |
| IE | Ireland |
| IL | Israel |
| IS | Iceland |
| IT | Italy |
| JP | Japan |
| KE | Kenya |
| KG | Kyrgyzstan |
| KP | Democratic People's Republic of Korea |
| KR | Republic of Korea |
| KZ | Kazakstan |
| LC | Saint Lucia |
| LI | Liechtenstein |
| LK | Sri Lanka |
| LR | Liberia |
| LS | Lesotho |
| LT | Lithuania |
| LU | Luxembourg |
| LV | Latvia |
| MC | Monaco |
| MD | Republic of Moldova |
| MG | Madagascar |
| MK | The former Yugoslav Republic of Macedonia |
| ML | Mali |
| MN | Mongolia |
| MR | Mauritania |
| MW | Malawi |
| MX | Mexico |
| NE | Niger |
| NL | Netherlands |
| NO | Norway |
| NZ | New Zealand |
| PL | Poland |
| PT | Portugal |
| RO | Romania |
| RU | Russian Federation |
| SD | Sudan |
| SE | Sweden |
| SG | Singapore |
| SI | Slovenia |
| SK | Slovakia |
| SN | Senegal |
| SZ | Swaziland |
| TD | Chad |
| TG | Togo |
| TJ | Tajikistan |
| TM | Turkmenistan |
| TR | Turkey |
| TT | Trinidad and Tobago |
| UA | Ukraine |
| UG | Uganda |
| US | United States of America |
| UZ | Uzbekistan |
| VN | Viet Nam |
| YU | Yugoslavia |
| ZW | Zimbabwe |

# Immuno-Magnetic cell separation used in identification of genes associated with site-preferenced cancer methastasis formation.

The object of the present invention is to provide a novel approach for detecting new genes with site-specific expression patterns in tumor cells residing in different tissues.

It is well known that many cancer types show typical patterns of spread, in that metastases appear preferably in certain tissues or organs, often in an orderly fashion. Thus, breast cancer metastases usually first develop in axillary lymph nodes, whereas bone marrow/bone metastases represent the first and most common (50%) site of distant spread. Of other tissues, liver, lung and the central nervous system (up to 20%) become hosts of breast cancer metastases. Similarly, prostate cancer gives skeletal metastases; colon cancer spreads to lymph nodes and liver; osteosarcoma to lung; and malignant melanoma to lymph nodes, liver, lung and brain. Very little is known about the factors that determine such tissue-preferenced cancer spread, but specific characteristics of the tumor cells are certainly involved, by e.g. enabling the tumor cells to home in the target organ, move to and invade the host tissue, respond to local growth factors, induce angiogenesis, or react in hitherto unknown ways. These characteristics must be associated with expression of specific proteins expressed by known or unknown genes. To identify such genes may, therefore, be of great importance in the understanding of the mechanisms of metastasis and thereby provide new leads or clues for diagnosis and therapy.

Several methods are being used in the search for genes that are highly expressed in certain populations of cells and not in other, including procedures such as subtractive hybridization cloning and the use of the differential display approach. Most such cloning projects have involved the use of *in vitro* cell lines or clones as starting material. However, it is known that cell lines may differ significantly from the tumor cells from which they originated, and that *in vitro* culture conditions may up- or down-regulate the expression of genes involved in deciding the ability of the cells to invade extracellular matrices and stromal tissue as well as their overall metastatic capacity.

A logical alternative to cell lines for comparing the expression of gene transcripts or proteins of interest would be to use specimens of tumor tissue from primary tumors and metastases from patients. Such an approach does, however, include several possibilities for error and also technical difficulties. When using tumor cells from different patients the expression of genes characteristic of each

individual must be subtracted before the patterns of gene expression related to the objective of the study are compared. This adds to the immense complexity of such gene cloning. It would be advantageous, therefore, to be able to compare expression patterns in cancer cells obtained from tumor manifestations located at different sites in one and the same individual. This may be possible by collecting tumor tissue from both the primary tumor and overt metastases detected and removed at surgery, and/or by surgery or biopsy of recurrent disease, or from secondary tumors in patients with progressive disease who have or have not received other treatment modalities after primary surgery.

In any gene cloning project it is important to work with as pure populations of target cells as possible, attempting to avoid irrelevant signals from non-target cells blurring the expression patterns to be compared. In specimens from solid tumors this is difficult to achieve, as in surgical specimens and biopsies the tumor cells will be mixed with normal fibrous tissue, including stromal and endothelial cells, that conventional methods of tissue preparation cannot satisfactorily remove. In hematological cancers, the malignant cells share determinants of a corresponding subpopulation of normal cells, preventing separation of the two types of cells.

In attempts to identify genes that are involved in the early stages of tumor dissemination, it would be important to obtain tumor cells from the relevant sites when the size of the secondary tumors is small, or if possible even from subclinical tumor foci or cells. One example would be malignant cells present in blood or in bone marrow before conventional diagnostic measures can demonstrate solid manifestation of metastasis. Another example would be cancer cells present in cerebrospinal fluid, in urine, or in effusions in pleural and abdominal cavities before conventional morphological procedures can detect such cells. Moreover, at primary surgery or if suspected for metastatic spread, lymph nodes are often removed because they are enlarged. Morphological examination may, however, still be negative in cases where a limited number of tumor cells still might be present. In all these examples the present invention describes a means of detecting and selecting the target tumor cells for gene cloning purposes.

In addition to using cancer cells obtained from different tissues or organs in patients, the invention also describes another way of obtaining metastatic human tumor cells for use in the search for genes with site-specific expression. Cells from several human tumors have been grown *in vitro* or in immunodeficient animals *in vivo*, and such cells have been used to establish experimental metastasis models or models in which the cells can be grown orthotopically, i.e. in the clinically relevant

tissues of origin; malignant melanoma in the skin; osteosarcoma in bone; colorectal cancer in the bowel wall; breast cancer in the mammary fat pad, etc. In experimental metastasis models different patterns of tumor spread can be seen, depending on the cell line, the route of cell injection, and the type of host used. Commonly the metastasis patterns simulate that of the corresponding tumor type in the clinic. By using such models it becomes possible also to obtain tumor cells from metastatic sites not usually accessible in patients, such as the spinal cord and the brain tissue. Again, for gene cloning purposes it would be important to select the human tumor cells from the animal cells to avoid problems with genes expressed in the normal host cells.

Previously it has been impossible to perform meaningful gene cloning experiments on specimens of solid tumors and metastases and on malignant cells in blood and bone marrow with the object of identifying genes with site-specific expression. This is because a considerable fraction of solid tumors and metastases is not malignant cells but connective tissue supporting and growing in between the malignant cells, and which also contains blood vessels that provide the necessary supply of nutritional factors and oxygen to the tumor. It has not been regarded as possible to adequately separate tumor cells from the normal cells without including an intermediate step of culturing the cells *in vitro*, involving manipulations to get rid of the normal cells. However, such *in vitro* cultivation would result in a selection of subpopulation of tumor cells in an environment quite different from the situation *in vivo*, thereby inducing significant changes in gene expression patterns. Therefore, for the purpose of identifying genes associated with the metastatic process, cultivation of tumor cells from solid metastases cannot be used. Moreover, it has not been regarded of interest to persons known in the field of gene cloning to compare gene expression patterns in malignant cells in untreated solid primary and metastatic tumors. Furthermore, in samples of blood and bone marrow tumor cells, if at all present, constitute a very low fraction of the total number of nucleated cells, and the malignant cells in blood and bone marrow have not been regarded of interest for gene cloning attempts. This is because the tumor cells could not be adequately separated from normal cells, and importantly also because people known in the art of gene cloning have not expected that such malignant cells were sufficiently different in gene expression patterns from those in the parent tumors.

Cancer cells isolated from human tumors metastasizing to different and clinically relevant target tissues and organs in immunodeficient animals have not been used in attempts to identify tumor-associated genes with site-specific expression, simply

because such human tumor models are very rare, but importantly also because of the lack of methods to obtain pure populations of cancer cells.

The object of the present invention is therefore to provide a method by which target cells can be separated from a cell population in order to identify the gene sequences from the target cells in a specific cell population environment.

This object has been obtained by the present invention characterized by the enclosed claims.

Since the object of the invention is to identify genes expressed specifically during early stages of tumor dissimination, the solid tumors and metastases should be small in size, and the malignant cells in blood and bone marrow should represent so-called micrometastatic disease, i.e. a limited a number of tumor cells should be present. If possible, such tumor cells should be collected even in cases where said cells cannot be detected by conventional morphological examinations or with diagnostic procedures such as radiology and magnetic resonance imaging. Evidently, since such cells have not been recognizable they have not been of interest for gene cloning purposes. The use of immunomagnetic techniques permits isolation of said malignant cells even when present in low numbers. Various methods for amplifying DNA and RNA sequences make gene cloning possible on such low numbers of malignant cells, provided that this cell population is sufficiently pure.

The positive selection of target tumor cells can be obtained by the use of per se known techniques, as described in patent application PCT/NO93/00136 (WO 94/07139) and in PCT/NO95/00052. Since in the present case the purity of the final target cell population is important, the immunomagnetic selection process may be performed more than once, or may be performed as a combination of positive (with target-cell recognizing monoclonal antibodies) and negative (with antibodies that bind to unwanted cells) selection. These techniques have been successfully used to isolate target cells from blood, bone marrow, malignant effusions, and from single cell suspensions prepared from solid tumor tissue of primary tumors and lymph node and other metastases. Similarly, the selection of human malignant cells from normal stromal or hematopoietic cells in animal hosts has also been demonstrated, even in cases where the tumor cells resided in bone, bone marrow, the spinal cord or in brain tissue. Since one objective would be to search for genes with products involved in the early stages of metastasis formation it may happen that only relatively few tumor cells can be obtained, the purity of

which is particularly important. With this approach it is possible to obtain a better purified target cell population from patients and animal hosts than by any other known technique, providing unique possibilities for cloning genes with site-specific expression.

The next step of the invention involves the use of known gene cloning procedures, such as the use of the differential display procedure first described by Liang, A. and Pardee, A.B. (Science, Vol 257, 967-971, 1992). In this method, polymerase chain reactions are performed with enzymes and primers that give reverse transcription and random amplification of gene transcripts present in the target cells. Resulting cDNA fragments from the cell populations to be compared are thereafter studied on a sequencing gel and the site-specific fragments extracted and sequenced. Gene fragments of interest can then be further studied, including examination of their expression patterns in material similar to that used for cloning. Again, having access to purified tumor cell populations without irrelevant non-target cells interfering with the results is very important. The possibility of using human tumor cells isolated from metastasis models in immunodeficient animals is advantageous also because the models provide a reliable and continuous source of target tumor cells for extended studies. It is also important that the target cells, either from patients or from animal models, are isolated and treated for DNA and RNA studies directly and very rapidly to avoid unwanted alterations in gene expression not related to the objective of identifying genes with site-specific expression.

Different methods used for cloning new genes have their inherent limitations. The differensial display-methods based on polymerase chain reaction techniques may suffer from problems related to representativity and reproducability. With our approach we have consequently included steps aimed at minimizing such problems. This has been achieved mainly by the use of the immunobead selection technique which makes it possible to study specifically biologically representative cells, not only for the first differential display step, but also in the steps where the candidate genes are examined for expression levels in relevant cells and tissues.

Example 1:

Tumor cells from the primary tumor and axillary lymph nodes from a breast cancer patient were prepared by physical and enzymatic methods to obtain a single cell suspension. A bone marrow sample (50 ml) was aspirated and a peripheral blood sample was obtained by venous puncture, and the mononuclear cells from both

occasions were isolated on Lymphoprep (Nycomed Pharma, Oslo, Norway). The cell suspensions were independently incubated with the MOC-31 and BM7 monoclonal antibodies, recognizing a pan-epithelial antigen and the MUC-1 gene product, respectively. After washing and incubation Dynabeads M-450 SAM SD (Dynal, Oslo, Norway), which bind to the Fc-region of the primary antibodies, were added. Tumor cells with bound antibody-Dynabeads M-450 SAM SD complex could thereafter be isolated from the normal mononuclear cells by the use of a strong magnet. The nature of the selected cells was confirmed microscopically. From the different cell populations RNA was extracted and the material subjected to the differential display cloning procedure (Liang and Pardee, 1992), in which partial cDNA-sequences from mRNA subpopulations obtained by reverse transcription were amplified by polymerase chain reaction. Comparison of cDNA-fragments from the various tumor cell populations were compared on a sequencing gel, and fragments specifically expressed in cells obtained from one of the sites were extracted and sequenced. Among a number of interesting gene sequences with specific expression either in tumor cells isolated, with our magnetic immunobead technology, from bone marrow or in tumor cells immunomagnetically isolated from lymph node metastases, we have found one cell cycle related transcription factor, one oncogene product, in addition to genes not yet identified. The expression of the two identified gene sequences in biologically relevant model systems and clinical material is presently being analyzed.

Example 2:

In this example, cells from a model for experimental metastasis of a human breast cancer were used. In athymic, nude rats injection of MA-11 human breast cancer cells into the cisterna magna (CM) results in leptomeningeal spread and growth of the malignant cells. Moreover, MA-11 cells injected into the left cardiac ventricle (LV) form metastases in the spinal cord which result in the development of hind leg paralysis in the animals after approximately 35 days. Tumor cells from both locations were obtained by mincing the host tissue and preparing single cell suspensions, and the immunobead technique described under example 1 was used to positively select the malignant cells. Thereafter, RNA extraction of cells from the two sites, together with *in vitro* cultured MA-11 cells was performed and the material similarly subjected to the differential display cloning procedure as already described. In addition, mRNA extracted from relevant normal tissues in the rats were included as controls. It should be noted that the MA-11 cell line was established from micrometastatic tumor cells isolates with the immunomagnetic method from the bone marrow of a stage II breast cancer patient. Several candidate fragments specific for the cells growing in the leptomeninges or as metastases to

the spinal cord have been detected. Sequence analysis showed that these fragments represent both novel and known genes. Among the fragments confirmed as differentially expressed in MA-11 cells selected from metastatic cell, four candidate genes have so far been examined in more detail. Two of these, termed LV1 and LV12 are particularly interesting. LV1 shows very high expression selectivity in tumor cells from spinal cord metastases, whereas LV12 is donwregulated in leptomeningeally growing cells. LV1 is found to be upregulated in a number of cell lines known to have a high capacity to form experimental metastasis in immunodeficient animals. In a panel of primary tumours from breast cancer pateients, low expression of LV12 was related to short survival of the corresponding patients. Altogether the results show that LV1 mRNA seems to be upregulated in highly metastatic cells, and LV12 mRNA level was inversely correlated to progression and metastasis of breast cancer cells. Both genes are novel. In addition, a third promising candidate gene, CM13, is also a novel gene upregulated in tumor cells growing in the leptomeninges. Several other candidates are still to be further analyzed. For both LV1 and LV12 cDNA full length cloning has been initiated.

Example 3:

In a similar model to that described in example 2, MT1 human mammary cancer cells give rise to growth in the leptomeninges after CM injection, whereas LV injected cells metastasize to the bone marrow and bone in the vertebra of the spine and in the long bones. Cells from these two locations as well as *in vitro* grown cells have been isolated and RNA from the different cell populations has been extracted. Similar cloning procedures to those described in example 2 can be applied to this material.

Example 4:

Tumor cells isolated by the technique described in example 1 have been isolated from other breast cancer patients, from patients with colorectal cancers where the tumor cells were isolated from primary and recurrent tumors, from lymph nodes and liver metastasis, and blood and bone marrow samples, and from prostate cancer patients with cells selected from primary or recurrent tumor specimens, from lymph nodes in addition to specimens from blood and bone marrow. The isolated cells can be used for gene cloning purposes.

In addition to the use of the material described in examples 1-4 for gene cloning, it can be used for examining the expression patterns of all gene sequences of sufficient promise.

PATENT CLAIMS

1. Method for identifying genes with site-specific or site preferred expression in specific target cells present in a cell environment different or not from that of their origin,
**characterized in** that the target cells initially are detected and isolated by repeated immuno-magnetic procedures in order to obtain up to 100% specific target cells before exposing the said target cells to known cell cloning procedures.

2. Method according to claim 1,
**characterized in** that the used target cells are malignant cells obtained from solid primary or recurrent tumors; and/or from metastases from such tumors to lymph nodes; and/or blood; and/or bone marrow; and/or bone tissue; and/or liver; and/or lungs; and/or central nervous system; and/or malignant pleural effusions and ascites, urine; and/or cerebral spinal fluid; and/or other organ sites.

3. Method according to claims 1-2,
**characterized in** that the malignant cells are isolated from single cell suspensions prepared from solid tumor manifestations; and/or from mononuclear cell fractions obtained from bone marrow or blood samples; and/or from cells present in other body fluids.

4. Method according to claims 1-2,
**characterized in** that the malignant cells used are *in vitro* cultivated human tumor cells; and/or human tumor cells grown in specific tissues in immunodeficient animals; and/or experimental human tumor metastases in such animals.

5. Method according to claims 1-4,
**characterized in** that RNA and/or DNA are extracted from the isolated cells.

6. Method according to claim 5,
**characterized in** that the extracted nucleic acids are used for gene cloning purposes.

7. Method according to the above claims,
**characterized in** that the said gene cloning method is the differential display or the subtractive hybridization approaches, or any other procedure that can be used to identify genes with differential expression.

8. Method according to claim 7,
**characterized in** that amplified cDNAs obtained from malignant cells selected from different sites are studied and compared on sequencing gels, and where those with interesting site-specific or site-preferenced patterns are sequenced and identified.

9. Method according to claim 8,
**characterized** in that the expression patterns of identified gene sequences are studied on material obtained from all relevant tumor cell sites described in claims 1-4.

10. Method according to the preceding claims,
**characterized in** that previously unknown genes identified in preceding claims are used for gene therapy purposes, and/or as targets for procedures aimed at altering or inactivating the genes or their products.

11. Use of the method according to claim 1, to obtain specific gene sequences and their expression products in target cells present in cell environments different or not from their origin.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/68, C12N 5/00, G01N 33/543
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CANCERLIT, EMBASE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9524648 A1 (FODSTAD), 14 Sept 1995 (14.09.95), page 4, line 2 - page 8, line 9 -- | 1-9 |
| X | WO 9407139 A1 (FODSTAD), 31 March 1994 (31.03.94), See pages 4-8 and 16 and claim 14 -- | 1-9 |
| X | National Library of Medicine, file Medline, Medline accession no. 95268055, Van Epps DE et al: "Harvesting, characterization, and culture of CD34+ cells from human bone marrow, peripheral blood, and cord blood", Blood Cells 1994;20(2-3):411-23 -- | 1-6 |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 July 1997 | 08 -07- 1997 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Carl-Olof Gustafsson<br>Telephone No. +46 8 782 25 00 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Dialog Information Service, file 154, Medline, Dialog accession no. 07911215, Medline accession no. 94231000, Wixler V. et al: "Isolation and quantification of class 1 MHC gene mutants in mouse T cells by immunoselection with a magnetic cell sorter (MACS)",J Immunol Methods May 2 1994, 171 (1) p121-30 | 1,5,6 |
| | -- | |
| X | Dialog Information Service, file 154, Medline, Dialog accession no. 06887713, Medline accession no. 92084142, Kappes DJ et al: "A novel method for generating stabel high-level transfectants involving direct immunomagnetic selection for cell-surface epitopes: exprression of HLA class-II genes in HeLa cells", Gene Dec 15 1991, 108 (2) 245-52 | 1,4-6 |
| | -- | |
| X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01195522, Cancerlit accession no. 96133084, Yaremko ML et al: "Immunomagnetic separation can enrich fixed solid tumors for epithelial cells", Am J Pathol; 148(1):95-104 1996 | 1-3,5,7 |
| | -- | |
| X | National Library of Medicine, file Medline, Medline accession no. 95325659, Griwatz C et al: "An immunological enrichment method for epithelial cells from peripheral blood", J Immunol Methods 1995 Jun 28;183(2):251-65 | 1-9 |
| | -- | |
| P,X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01193425, Cancerlit accession no. 96709393, Ree AH et al: "Identification of site specific gene expression in metastatic breast cancer cells (Meeting abstract)", Proc Annu Meet Am Assoc Cancer Res; 37:A607 1996 | 1-9 |
| | -- | |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01192976, Cancerlit accession no. 96653514, Brandt B. et al: "Isolation of single cancer cells and coherent cell sheets from peripheral blood of cancer patients with reference to shed cytokeratin 8/18 and erbB-2 oncoprotein fragments (Meeting abstract)", Proc Annu Meet Am Assoc Cancer Res; 37:A555 1996 | 1-9 |
| | -- | |
| A | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 00864158, Cancerlit accession no. 92257480, Gazitt Y et al: "Expression of N-Myc, and MDR-1 Proteins in Newly Established Neuroblastoma Cell Lines: A Study by Immunofluorescence Staining and Flow Cytometry", Cancer Res; 52(10):2957-65 1992 | 1-9 |
| | -- | |
| | -------- | |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NO 97/00083

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9524648 A1 | 14/09/95 | AU 2086495 A | 25/09/95 |
| | | CA 2185128 A | 14/09/95 |
| | | EP 0749580 A | 27/12/96 |
| | | FI 963533 A | 07/11/96 |
| | | NO 180658 B | 10/02/97 |
| | | NO 940866 A | 11/09/95 |
| | | PL 316209 A | 23/12/96 |
| WO 9407139 A1 | 31/03/94 | AU 2593192 A | 12/04/94 |
| | | AU 4836393 A | 12/04/94 |
| | | CA 2144328 A | 31/03/94 |
| | | EP 0660930 A | 05/07/95 |
| | | FI 951161 A | 09/05/95 |
| | | HU 73741 A | 30/09/96 |
| | | HU 9500723 D | 00/00/00 |
| | | JP 8501390 T | 13/02/96 |
| | | NO 950918 A | 10/05/95 |
| | | PL 308109 A | 24/07/95 |
| | | SK 32995 A | 11/10/95 |
| | | WO 9407138 A | 31/03/94 |

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C12N 5/00, G01N 33/543 — A1

(11) International Publication Number: WO 97/36004

(43) International Publication Date: 2 October 1997 (02.10.97)

(21) International Application Number: PCT/NO97/00083

(22) International Filing Date: 25 March 1997 (25.03.97)

(30) Priority Data:
961221 26 March 1996 (26.03.96) NO

(71)(72) Applicant and Inventor: FODSTAD, Øystein [NO/NO]; Frits Kiærs vei 28, N-0383 Oslo (NO).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ENGEBRÅTEN, Olav [NO/NO]; Haneborgsveien 43A, N-1470 Lørenskog (NO). REE, Anne, Hansen [NO/NO]; Kirkeveien 87A, N-1344 Haslum (NO). HOVIG, Johannes, Eivind [NO/NO]; Sognsveien 31, N-0851 Oslo (NO).

(74) Agent: ONSAGERS PATENTKONTOR A/S; P.O. Box 265 Sentrum, N-0103 Oslo (NO).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: IMMUNO-MAGNETIC CELL SEPARATION USED IN IDENTIFICATION OF GENES ASSOCIATED WITH SITE-PREFERENCED CANCER METHASTASIS FORMATION

(57) Abstract

Method for identifying genes with site specific or site preferred expression in target cells that are initially detected and isolated by repeated immuno-magnetic procedures. The purified target cells are then exposed to known cloning procedures. Preferred target cells are malignant cells, e.g. metastatic cells. Gene cloning methods may include the differential display or substractive hybridization approaches.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/68, C12N 5/00, G01N 33/543
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CANCERLIT, EMBASE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9524648 A1 (FODSTAD), 14 Sept 1995 (14.09.95), page 4, line 2 - page 8, line 9 | 1-9 |
| X | WO 9407139 A1 (FODSTAD), 31 March 1994 (31.03.94), See pages 4-8 and 16 and claim 14 | 1-9 |
| X | National Library of Medicine, file Medline, Medline accession no. 95268055, Van Epps DE et al: "Harvesting, characterization, and culture of CD34+ cells from human bone marrow, peripheral blood, and cord blood", Blood Cells 1994;20(2-3):411-23 | 1-6 |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 July 1997 | 08 -07- 1997 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Carl-Olof Gustafsson<br>Telephone No. +46 8 782 25 00 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Dialog Information Service, file 154, Medline, Dialog accession no. 07911215, Medline accession no. 94231000, Wixler V. et al: "Isolation and quantification of class 1 MHC gene mutants in mouse T cells by immunoselection with a magnetic cell sorter (MACS)",J Immunol Methods May 2 1994, 171 (1) p121-30 | 1,5,6 |
| X | Dialog Information Service, file 154, Medline, Dialog accession no. 06887713, Medline accession no. 92084142, Kappes DJ et al: "A novel method for generating stabel high-level transfectants involving direct immunomagnetic selection for cell-surface epitopes: exprression of HLA class-II genes in HeLa cells", Gene Dec 15 1991, 108 (2) 245-52 | 1,4-6 |
| X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01195522, Cancerlit accession no. 96133084, Yaremko ML et al: "Immunomagnetic separation can enrich fixed solid tumors for epithelial cells", Am J Pathol; 148(1):95-104 1996 | 1-3,5,7 |
| X | National Library of Medicine, file Medline, Medline accession no. 95325659, Griwatz C et al: "An immunological enrichment method for epithelial cells from peripheral blood", J Immunol Methods 1995 Jun 28;183(2):251-65 | 1-9 |
| P,X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01193425, Cancerlit accession no. 96709393, Ree AH et al: "Identification of site specific gene expression in metastatic breast cancer cells (Meeting abstract)", Proc Annu Meet Am Assoc Cancer Res; 37:A607 1996 | 1-9 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00083

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 01192976, Cancerlit accession no. 96653514, Brandt B. et al: "Isolation of single cancer cells and coherent cell sheets from peripheral blood of cancer patients with reference to shed cytokeratin 8/18 and erbB-2 oncoprotein fragments (Meeting abstract)", Proc Annu Meet Am Assoc Cancer Res; 37:A555 1996 | 1-9 |
| | -- | |
| A | Dialog Information Service, file 159, Cancerlit, Dialog accession no. 00864158, Cancerlit accession no. 92257480, Gazitt Y et al: "Expression of N-Myc, and MDR-1 Proteins in Newly Established Neuroblastoma Cell Lines: A Study by Immunofluorescence Staining and Flow Cytometry", Cancer Res; 52(10):2957-65 1992 | 1-9 |
| | -- | |
| | -------- | |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NO 97/00083

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9524648 A1 | 14/09/95 | AU 2086495 A | 25/09/95 |
| | | CA 2185128 A | 14/09/95 |
| | | EP 0749580 A | 27/12/96 |
| | | FI 963533 A | 07/11/96 |
| | | NO 180658 B | 10/02/97 |
| | | NO 940866 A | 11/09/95 |
| | | PL 316209 A | 23/12/96 |
| WO 9407139 A1 | 31/03/94 | AU 2593192 A | 12/04/94 |
| | | AU 4836393 A | 12/04/94 |
| | | CA 2144328 A | 31/03/94 |
| | | EP 0660930 A | 05/07/95 |
| | | FI 951161 A | 09/05/95 |
| | | HU 73741 A | 30/09/96 |
| | | HU 9500723 D | 00/00/00 |
| | | JP 8501390 T | 13/02/96 |
| | | NO 950918 A | 10/05/95 |
| | | PL 308109 A | 24/07/95 |
| | | SK 32995 A | 11/10/95 |
| | | WO 9407138 A | 31/03/94 |

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| Applicant's or agent's file reference | FOR FURTHER ACTION | See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416) |
|---|---|---|
| Fod4 im | | |
| International application No. | International filing date *(day/month/year)* | Priority date *(day/month/year)* |
| PCT/NO97/00083 | 25/03/1997 | 26/03/1996 |
| International Patent Classification (IPC) or national classification and IPC | | |
| C12Q1/68 | | |
| Applicant | | |
| FODSTAD, Oystein et al | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

| | | |
|---|---|---|
| I | ☒ | Basis of the report |
| II | ☐ | Priority |
| III | ☐ | Non-establishment of opinion with regard to novelty, inventive step and industrial applicability |
| IV | ☐ | Lack of unity of invention |
| V | ☒ | Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement |
| VI | ☒ | Certain documents cited |
| VII | ☐ | Certain defects in the international application |
| VIII | ☐ | Certain observations on the international application |

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 16/10/1997 | 29.04.98 |
| Name and mailing address of the IPEA/ | Authorized officer |
| European Patent Office<br>D-80298 Munich<br>Tel. (+49-89) 2399-0. Tx: 523656 epmu d<br>Fax: (+49-89) 2399-4465 | Goetz, M<br>Telephone No. (+49-89) 2399-8697 |

# NTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NO97/00083

## I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

| | | | | | |
|---|---|---|---|---|---|
| 1-7 | as originally filed | | | | |

**Claims, No.:**

| | | | | | |
|---|---|---|---|---|---|
| 1-11 | as received on | 09/04/1998 | with letter of | 07/04/1998 | |

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 1-11 |
| | No: | Claims | |
| Inventive step (IS) | Yes: | Claims | 1-11 |
| | No: | Claims | |
| Industrial applicability (IA) | Yes: | Claims | 1-11 |
| | No: | Claims | |

2. Citations and explanations

**see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**see separate sheet**

## REASONED STATEMENT

1. None of the documents cited in the IPER relates to the use of the combined techniques

   a) immuno-magnetic techniques for the isolation of specific target cells,
   b) cell cloning procedures,

   for identifying unknown genes with site-specific or site-preferred expression at the mRNA levels.

   Hence, claims 1 - 11 meet the novelty requirements pursuant to Art. 33(2) PCT.

2. Although the manipulative technique listed under a) and b) above are known from the prior art as represented by

   **D1 = WO95/24648**
   **D2 = WO94/07139**

   (see **D1**, page 5/lines 14 - 16, page 15/lines 20-26, claims 1 - 13 and 16; see **D2**, page 3/last paragraph, claims 1 - 14), and e.g. **Science 257, 1992, p. 967 - 971**, (cited in the application), the present inventors apparently were the first to establish that the preferential appearance of metastatic tumor cells in certain tissues is due to an up- and down-regulation mechanism of specific genes in the tumor cells, reflected by differential mRNA expression.

   Hence, the use of known techniques a) and b) for identifying unknown genes participating in such mechanisms involves an inventive step.

   Claims 1 - 11 therefore meet the requirements pursuant to Art. 33(3) PCT.

→

## CERTAIN DOCUMENTS

As the present application is fully entitled to the claimed priority date of 26/03/96, the interfering documents, i.e.

**Proc.Annu.Meet.Am.Assoc.Cancer Res., 37:A607, 1996**
and **Proc.Annu.Meet.Am.Assoc.Cancer Res., 37:A555, 1996**

are not considered to represent state of the art relevant to presently claimed subject-matter.

PATENT CLAIMS

1. Method for identifying genes with site-specific or site preferred expression in specific target cells present in a cell environment different or not from that of their origin,
**characterized in** that the target cells initially are detected and isolated by repeated immuno-magnetic procedures in order to obtain up to 100% specific target cells before exposing the said target cells to known gene cloning procedures, wherein unknown genes with differences in levels of mRNA expressions in the target cells isolated from different tissues, are compared.

2. Method according to claim 1,
**characterized in** that the used target cells are malignant cells obtained from solid primary or recurrent tumors; and/or from metastases from such tumors to lymph nodes; and/or blood; and/or bone marrow; and/or bone tissue; and/or liver; and/or lungs; and/or central nervous system; and/or malignant pleural effusions and ascites, urine; and/or cerebral spinal fluid; and/or other organ sites.

3. Method according to claims 1-2,
**characterized in** that the malignant cells are isolated from single cell suspensions prepared from solid tumor manifestations; and/or from mononuclear cell fractions obtained from bone marrow or blood samples; and/or from cells present in other body fluids.

4. Method according to claims 1-2,
**characterized in** that the malignant cells used are *in vitro* cultivated human tumor cells; and/or human tumor cells grown in specific tissues in immunodeficient animals; and/or experimental human tumor metastases in such animals.

5. Method according to claims 1-4,
**characterized in** that RNA and/or DNA are extracted from the isolated cells.

6. Method according to claim 5,
**characterized in** that the extracted nucleic acids are used for gene cloning purposes.

7. Method according to the above claims,
**characterized in** that the said gene cloning method is the differential display or the subtractive hybridization approaches, or any other procedure that can be used to identify genes with differential expression.

8. Method according to claim 7,
**characterized in** that amplified cDNAs obtained from malignant cells selected from different sites are studied and compared on sequencing gels, and where those with interesting site-specific or site-preferenced patterns are sequenced and identified.

9. Method according to claim 8,
**characterized** in that the expression patterns of identified gene sequences are studied on material obtained from all relevant tumor cell sites described in claims 1-4.

10. Method according to the preceding claims,
**characterized in** that previously unknown genes identified in preceding claims are used for gene therapy purposes, and/or as targets for procedures aimed at altering or inactivating the genes or their products.

11. Use of the method according to claim 1, to obtain specific gene sequences and their expression products in target cells present in cell environments different or not from their origin.

# PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

## PCT

### NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

| | |
|---|---|
| **Date of mailing** (day/month/year)<br>30 October 1997 (30.10.97) | |
| **International application No.**<br>PCT/NO97/00083 | **Applicant's or agent's file reference**<br>Fod 4 HV |
| **International filing date** (day/month/year)<br>25 March 1997 (25.03.97) | **Priority date** (day/month/year)<br>26 March 1996 (26.03.96) |
| **Applicant**<br>FODSTAD, Øystein et al | |

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   16 October 1997 (16.10.97)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

**The International Bureau of WIPO**
**34, chemin des Colombettes**
**1211 Geneva 20, Switzerland**

Facsimile No.: (41-22) 740.14.35

Authorized officer

I. Britel

Telephone No.: (41-22) 338.83.38